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ISOLATION AND CLONING OF METHIONINASE GENE OF *PSEUDOMONAS PUTIDA*

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ABSTRACT: *Pseudomonas putida* is a Gram-negative, rod-shaped, saprotrophic soil bacterium. Based on 16S rRNA analysis, P. putida was taxonomically confirmed to be a *Pseudomonas* species. Faster detection of the causative Microbes and institution of proper therapy would help greatly in preventing septic complications. Recent advances in the field of Molecular Biology, including the amplification of genetic material by the Polymerase Chain Reaction (PCR) technologies have led to faster and more reliable microbial detection methods. Results would help in early detection of methionase gene in *Pseudomonas Putida*. In this work we have identified Methionase gene in *Pseudomonas Putida* and amplified using Polymerase Chain reaction.

Key words: 16sRNA, Methionase, PCR, *Pseudomonas Putida*

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INTRODUCTION

L-Methioninase is a pyridoxal-5′-phosphate-subordinate catalyst, catalyzes the γ-disposal of Lmethionine to produce α-ketobutyrate, methanethiol, and smelling salts and also the α, β-substitution and β-end of Ssubstituted L-cysteines (Tanaka et al., 1983). A noteworthy potential remedial utilization of chemicals is in the treatment of tumor. Along these lines, much consideration has been paid to L-methioninase which have exhibited antitumor viability in vitro and additionally in vivo (Kahraman et al., 2011; Tan et al., 1998). L-methioninase is one of couple of microbial catalysts with high remedial esteem since it was accounted for as a strong anticancer operator against different sorts of tumor cell lines Breast, Lung, Colon, Kidney and Glioblastoma (Kokkinakis et al., 2001; Tan et al., 1998). Numerous human growth cell lines and essential tumors have a flat out necessity for L-methionine as a fundamental amino corrosive, to survive and multiply (Anderson, 1998). Then again, typical cells are methionine free as they have dynamic methionine synthase. Along these lines, they can develop on a medium supplemented with homocysteine, vitamin B12 and folic corrosive rather than methionine. The nonappearance of methionine synthase in numerous tumors, in as opposed to the typical cells, mostly clarifies the failure of tumor cells to develop on homocystein (Kahraman et al., 2011 and Cellarier et al., 2003). Thus, methionine is the fundamental tumor particular focus for restorative systems. Subsequently, helpful abuse of L–methioninase to exhaust plasma methionine is by all accounts a promising system (Sharma et al., 2014; Sundar and Nellaiah, 2013; Pinnamaneni et al., 2012; Yoshioka et al., 1998). L-methioninase was widely described from different bacterial species particularly Pseudomonas. Sanitization of L-methioninase from Pseudomonas ovalis was completed by (Tanaka et al., 1976). They detailed that, the cleansed protein had an atomic weight of 43KDa.

A comparable compound from an alternate clone of Pseudomonas putida was detached and cleansed by (Ito et al., 1976). The purged chemical from Pseudomonas putida ICR 3460 was distributed by (Nakayama et al., 1984) has a sub-atomic weight 43KDa. Besides, immaculate L-methioninase was likewise acquired from different types of microorganisms, for example, Colstridiumsporogens (Kreis and Hession, 1973), Brevibacterium cloths (Pinnamaneni et al., 2012). Then again, a couple examines on the purging and portrayal of L-methioninase from growths, for example, Aspirgillusflvips announced by (El-Sayed, 2011), the cleaned catalyst had a sub-atomic mass 47 KDa. What's more, Purification and portrayal of this protein from the yeast was done by (Selim et al., 2015a).

METHODOLOGY

Isolation of Bacterial strains

The different soil samples containing the bacteria were collected from different areas of Vijayawada Corporation, Andhra Pradesh. The soil sample was collected from a depth of 15 inches into the soil where there was high moisture content, because the bacteria need moisture content for growth. The soil sample collected was dispensed in the sterile bags and sealed and the sample was brought to the laboratory.

Serial dilution technique

The bacterium *pseudomonas putida* was isolated from the soil samples collected from different areas of Vijayawada Corporation, Andhra Pradesh. The soil sample was subjected to serial dilution technique. Serial dilution involves repeatedly mixing known amounts of sample source with (sterilized) liquid. About 1 gm of soil was added to 10ml of distilled water gives a 10-fold dilution; 1ml added to 99ml gives a 100-fold dilution procedure. The serial dilution is a technique that allows the decrease in the no of the bacteria present as the dilution rete increase. The purpose of the dilution is to obtain pure isolated colonies, which was obtained on the dilution of the soil sample.

Selective media

A selective medium is the one that contains one or more agents that inhibit the growth of a certain microbe or microbes and thereby encourages, or selects the microbe of interest and allows it to grow. Selective media are very important in primary isolation of a specific type of microorganism from samples containing dozens of different spices- for example, from soil. They hasten isolation by suppressing the unwanted background organisms and favoring growth of the desired ones.

Pseudomonas CFC selective supplement Agar Medium

Pseudomonas CFC selective supplement agar Medium from HI-MEDIA is a selective medium in which the cetrimide and casein hydrolysate has been supplemented as a carbon source. The culture broth is inoculated in to the *Pseudomonas* CFC selective supplement agar Medium.

Identification of the Bacteria

The isolated bacterium was identified using different staining techniques, different biochemical and molecular techniques.

Simple staining

Bacteria are very small and transparent when observed with a wet mount preparation. In order to observe their cell characteristics, they need to be stained (Dyed). This method consists of preparing a smear that is air dried and heat fixed and adding a stain to the bacteria on the slide. The simple stain consists of one dye. The dye adheres to the cell wall and colours the cell making it easier to see. Basic strains, such as methylene blue, Gram saffranin or Gram crystal violet are used for staining the most bacteria. These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since the surface of most bacterial cells is negatively charged, these positively charged stains adhere readily to the cell surface.

Gram staining

Several different kinds of bacteria will be examined by gram staining, a method developed by Christian Gram in 1884 for categorizing bacteria on the basis of differences in cell wall structure. Gram positive (+) bacteria stain a dark purple color, while gran negative (-) bacteria stain a light red. Microscopy reveals at least two different types of cell wall structure: single and double. The walls of gram positive bacteria consists of a single, thick, continuous layer, whereas those of gram negative bacteria consists of at least two readily distinguishable layers, each considerably thinner than the wall of gram positive bacterium. In addition, chemical analyses reveal substantial differences in the chemical composition of the two kinds of cell walls. In preparing a gram stain, the first step stains both kinds of cell walls, gram positive and gram negative, with a deep purple dye complex. The next step exposes the cells to a decolorizing agent such as alcohol and then to the counterstaining red dye saffranin O. since the cell wall of the gram positive bacterium constitutes a barrier to the decolorizing agent, the gram positive bacterium remains purple, while the gram negative bacterium is decolorized and appears light red due to the color.

Identification of the isolated bacteria by Molecular Methods

The most powerful tool to identify the unknown bacteria is to sequence the DNA coding for 16s rRNA, since the 16s rRNA is encoded by the gene in the chromosome of the bacteria, So the gene coding for the 16s rRNA is amplified using the Polymerase Chain Reaction and the amplified product was confirmed by agarose gel electrophoresis and it has been subjected to sequencing and the sequence obtained has been compared with the sequence obtained from the Nucleotide database of National Center for Biotechnology Information (NCBI).

Nucleic acid sequencing

Genome structures can be directly compared by sequencing DNA and RNA. Since the 16s rRNA is the most conserved (least variable) gene in all cells. The ribosomes that are the work benches of the protein synthesis present in bacteria are of 70s ribosomes composed of two subunits 30s is the smaller subunit and 50s is the larger subunit. These two subunits bind only during the protein synthesis. The small ribosomal subunit (30s) contains the 16srRNA (s in 16s represents Svedberg units). The large ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Bacterial 16S, 23S and 5S rRNA genes are typically organized as a co-transcribed operon. There may be one or more copies of the operon dispersed in the genome. To infer relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. An example of genes in this category is those that define the ribosomal RNAs (rRNAs). Most prokaryotes have three rRNAs, called the 5S, 16S and 23S rRNA.

Ribosomal RNAs in Prokaryotes

5S located in the larger subunit of ribosome (50s) contains around 120 nucleotides, 16S located in the small subunit of ribosome (30s) contains around 1500 nucleotides and 23S located in the large subunit of ribosome contains around 2900 nucleotides.

The 5S has been extensively studied, but it is usually too small for reliable phylogenetic inference. The 16S and 23S rRNAs are sufficiently large to be useful. The 16s rDNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. These are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the 16S rDNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many species. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the National center for Biotechnology Information and the Ribosomal Database project. These sites also provide search algorithms to compare new sequences to their database.

Sequencing of the 16s rRNA Sequence

Some of the most powerful approaches to identify the unknown bacteria are the study of nucleic acids as it is the genes that are specific for specific protein. Comparisons of nucleic acids yield considerable information about true relatedness. These most recent molecular approaches have become increasingly important in prokaryotic taxonomy. Hence sequencing of the DNA coding for 16s rRNA can enable the identification of bacteria.

Procedure

The isolated DNA was amplified the region of the 16S rDNA gene of the isolated bacteria using Polymerase Chain Reaction (PCR). The amplified DNA was subjected to 1.5% of Agarose gel electrophoresis and basing on the size of the amplified fragment rDNA gene was confirmed. The DNA band of the amplified product was cut from gel, eluted and subjected for sequencing. The sequence so obtained was compared with the reported results with the public databases (NCBI) and the sequence of the unknown bacteria was determined.

The objective of this rDNA sequencing is to determine a new bacterial strain has been isolated basing on its taxonomy: whether it belongs to a known genus, and if not, to identify the new species within known genera and also to determine (as much as possible) if it is a new species or a new strain of an already known species.

Cloning of Methioninase gene from *Pseudomonas putida*

Isolation of DNA from *Pseudomonas putida*

1.5mlof overnight culture of *Pseudomonas putida* taken into 1.5ml centrifuge tube, Centrifuged at 8000rpm for 5min.Supernatant was discarded and briefly vortex pellet to dislodge.400µl of Lysis buffer [1.5M NaCl, 10mM Tris-HCL (pH 8.0), 5% SDS] was added to the dislodge pellet and incubated in water bath for 15min at 65^{o}C.The solution was cooled to room temperature and 130µl of potassium acetate (pH 5.2) was added and mixed. The solution was incubated for 5 minutes in room temperature. The sample was centrifuged at 1000rpm for 10mins. Clear supernatant was transferred into fresh 1.5ml of tube. Equal volume of isopropanol was added, gently inverted for 5 times and incubated for 10mins in room temperature. The sample was centrifuged at 10000rpm for 10mins. Supernatant was discarded and pellet is washed with 75% ethanol. The pellet was dried in room temperature for another 15mins. Pellet was dissolved in 100µl of TE buffer. To remove RNA from the preparation, 10µl of RNaseA (10mg/ml) was added and incubated at 37^{o}C for 1hour.

Purification of *Pseudomonas putida*DNA

5µl/ml RNase solution (the amount of RNase depends upon the RNA contamination) was added to the crude DNA and incubated at 37^{o}C for 45 minutes.To the above, 1 ml of TE saturated phenol was added, mixed the contents thoroughly and then centrifuged at 15,000x g at 4^{o}C.The upper aqueous phase was transferred to a fresh tube and added with equal volume of a mixture of phenol: chloroform: isoamylalcohol (25:24:1) to the solution and mixed thoroughly without vortexing.The contents were centrifuged at 15,000 x g at 4^{o}C for 5 minutes and transferred the upper aqueous phase to a fresh tube.And to the contents, an equal volume of Chloroform: Isoamylalcohol (24:1) was added and centrifuge at 15,000 x g at 4^{o}C for 5 minutes. This step is repeated until no precipitate is seen.To the 1/10th volume (of the aqueous phase) of 3M Sodium acetate (pH 5.2) was added, mixed the contents it and then added with twice its volume of chilled absolute ethanol. Mixed the contents thoroughly by inverting the tubes and incubate at -20^{o}C for one hour to get precipitate of the DNA.The contents were centrifuged at 15,000 x g for 5 minutes at 4^{o}C. The DNA was collected discarding the supernatant and the pellet was air dried to remove ethanol and dissolved in appropriate volume of TE buffer.

Quantification of Pseudomonas putida DNA

In this procedure, the DNA was diluted in water and absorbance was measured at 230, 260, 280 and 300nm. From this method an appropriate idea about the amount of protein contamination can be known but the drawback is that if there is RNA or small nucleic acid contamination, qualification of DNA would be wrong since absorption maxima of all nucleic acids are at 260nm. This method requires microgram amounts of DNA to ensure reliable readings.

Amplification of Methionasegene of *Pseudomonas putida*by Polymerase Chain Reaction (PCR)

The Metase gene of Pseudomonas putida was amplified in a Master cycler gradient programmed at 5 minutes. In polymerase Chain reaction, the specific primers were used to amplify the genomic sequence of the open reading frame of the gene. The master mix containing 10X Taq buffer, 10mM dNTPs, 25 mM of MgCl2, 1 U of Taq DNA polymerase, 1.5 µl of Reverse primer, 100 ng of Genomic DNA and PCR grade molecular water were made to the final volume 20 µl was used. Taq DNA polymerase initiates the replication of DNA fragments by using nucleotide base from dNTP mixture (A, T, G and C). Linear amplification was performed at 95^{o}C for 5 minutes, followed by 40 cycles of 30s of denaturation at 94^{0}C, 30s of annealing at 60^{0}C, and 3 minutes of polymerization at 72^{0}C and then 72^{0}C for 3 minutes. Exponential amplification was performed at 95^{0}C for 5 minutes, followed by 35 cycles of 30s of denaturation at 94^{0}C, 30s of annealing at 60^{0}C, and 2 minutes of polymerization at 72^{0}C for 10 minutes. The PCR products were analyzed by Agarose gel Electrophoresis on a 1.6 percent agarose gel, visualized under UV light, photographed and documented with an Alpha Imager (Alpha Innotech, California, and USA).

Agarose gel electrophoresis

Required amount of agarose (w/v) was weighed and melted in 1X TBE buffer (0.9M Tris-borate, 0.002 M EDTA, pH 8.2). Then, 1-2 µl ethidium bromide was added from the stock (10mg/ml H2). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TBE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out 60V (Sambrook et al., 1989).

Eluting DNA from agarose gel fragments

Ethidium bromide stained agarose gel was visualized under a transilluminator on low setting. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column.The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA.The eluent was checked using a transilluminator for the presence of ethidium bromide stained DNA.The eluted DNA was used directly in enzymatic reactions.This DNA fraction was now subjected for sequencing.

RESULTS AND DISCUSSION

Streak Plate Technique: Colour less colonies were observed over the medium (Fig 1).

Fig 1: Bacterial colonies

GRAM STAINING

Observation

On Gram staining pink colour rods were observed. Hence it is a Gram negative bacteria.From above observation it is said that is a Gram negative bacteria (Fig 2).

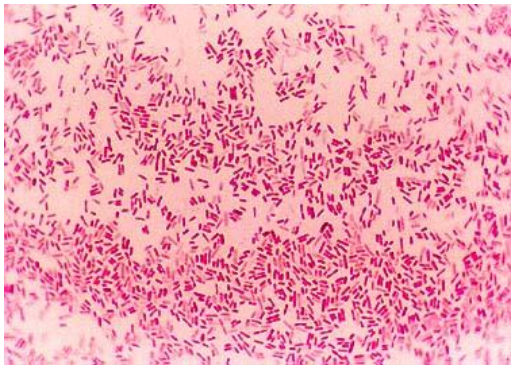

Fig 2: Gram staining

STARCH TEST

After 48 hrs of incubation it was observed that sugars that are glucose, sucrose and lactose were utilized by *organism* acid was produced in glucose, lactose and sucrose (Figure 3).Organism utilized all the three sugars and produced to the acid so it is positive.

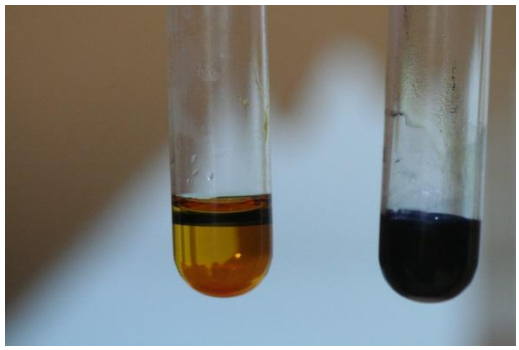

Fig 3: Fermentation of Carbohydrates

CATALASE ACTIVITY

After 48 hours of incubation when four drops of hydrogen peroxide was added to theslants slow appearance of gas bubbles was observed (Figure 4).After the addition of hydrogen peroxide gas bubbles were observed which is theindication of positive test.

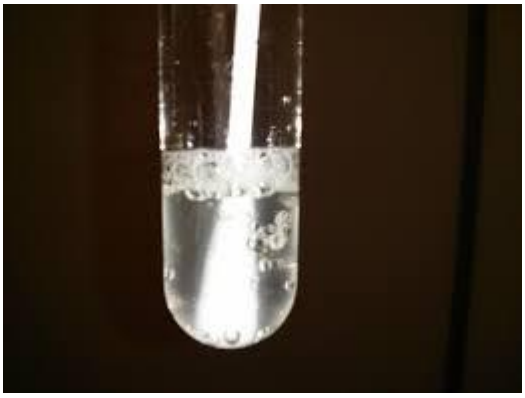

FIGURE 4: Catalase activity

HYDROGEN SULPHIDE PRODUCTION TEST

No black coloration along the line of stab inoculation was observed (Figure 5).Black coloration along the line of stab inoculation was not observed. Hence the organism may be H_2S negative.

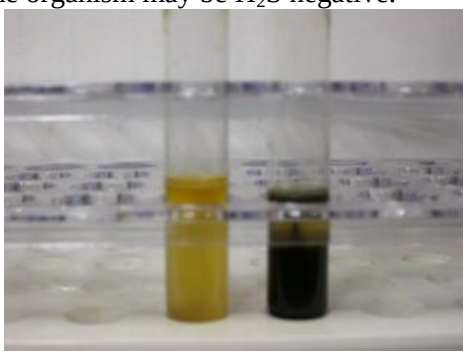

Figure 5:Hydrogen Sulphide test

INDOLE PRODUCTION TEST

Development of cherry (deep) red color in the top layer of the tube is not observed. As development of cherry red color is not observed in the top layer of the tube soit is negative test (Fig 6).

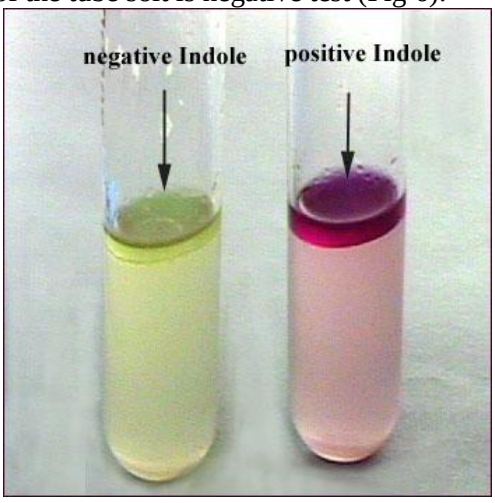

Fig 6: Indole Test

METHYL-RED AND VOGES-PROSKAUER TESTS

The tubes in which methyl red was added no red color was observed in the V-P test tubes when V-P reagents I & II were added red color was not observed (Figure 7 and 8).As in the methyl red test red color is observed hence, it is Negative test.

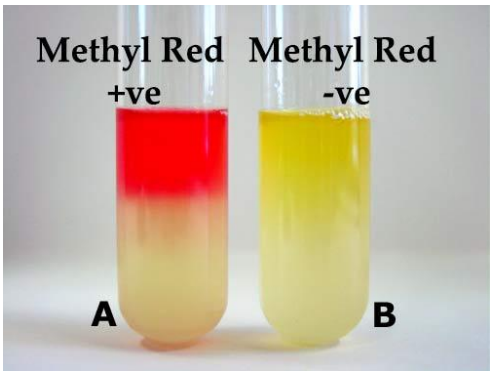

Fig 7: Methyl-Red test

VP TEST RESULT

In the VP test, red color is not observed hence, it is negative test (Fig 9).

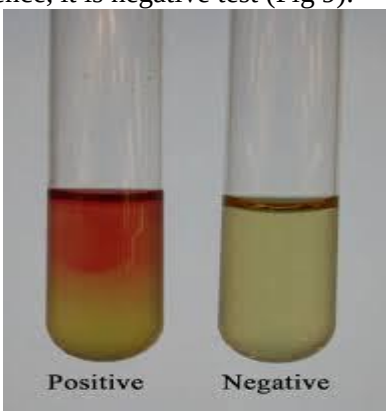

Figure 8:Voges-Proskeur Test

CITRATE UTILIZATION TEST

After 48 hours of incubation it was observed that there is change in the medium color.From the above observation it is positive to this test (Fig 9).

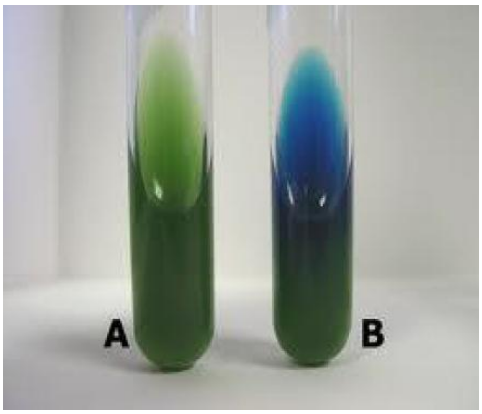

Figure 9: citrate test

UREASE TEST

After 48 hours of incubation it was observed that there is change in the medium.From the above observation it is shows positive test (Fig 10).

Figure 10: Urease test

Isolation of DNA and PCR

Following are the pictures taken of the PCR sample upon running the gel, PCR cycler (instrument used) and picture of me working in the lab. Based on the amplification result the organism was identified as *Pseudomonas Putida*(Fig 11).

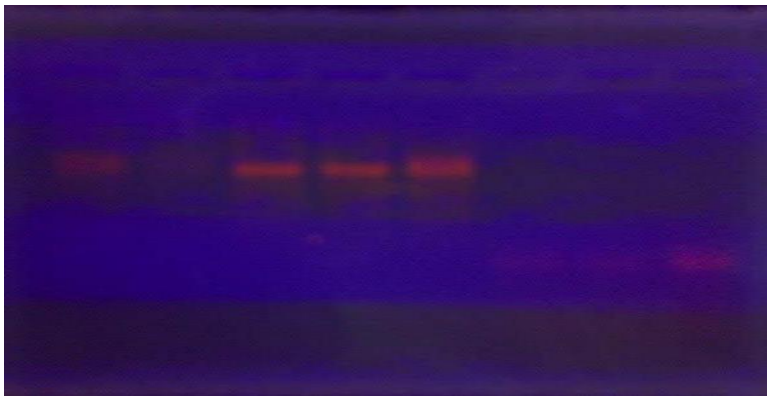

Fig 11: Genomic DNA

After running the PCR, we got the product of methioninase gene near 270bp region. That is, the fragment size after amplification is found to be 200bp. Since, this is a partial clone; efforts are underway to pull out the full length clone (Fig 12).

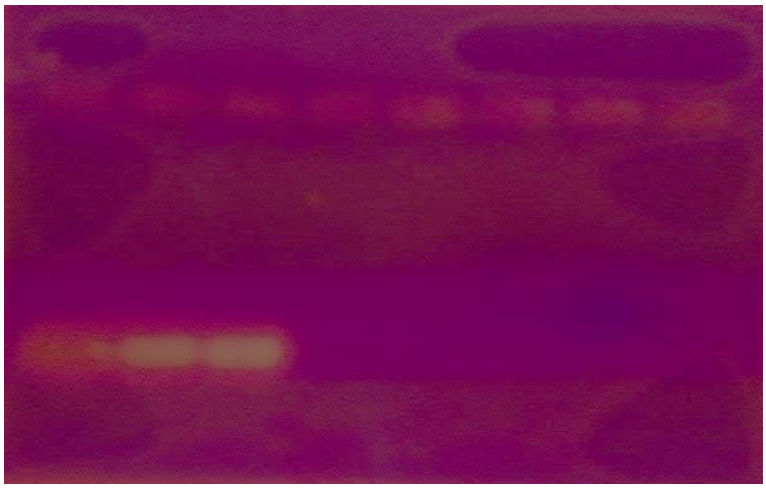

Fig 12: PCR

CONCLUSION

We can conclude that from the microbial and biochemical characterization of the test organism confirmed as Pseudomonas putida after molecular based techniques. The PCR product was further analysed by using RDP-Ribosome project for analysis. Based on the 16sRNA sequence the culture was identified as *Pseudomonas putida*.

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